Structure, Vol. 13, 1745–1754, December, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.str.2005.08.011

# Release of a Damaged Cofactor from a Coenzyme $B_{12}$-Dependent Enzyme: X-Ray Structures of Diol Dehydratase-Reactivating Factor

**Naoki Shibata,[1] Koichi Mori,[2] Naoki Hieda,[2] Yoshiki Higuchi,[1] Mamoru Yamanishi,[2] and Tetsuo Toraya[2,*]**
[1]Graduate School of Life Science
University of Hyogo
3-2-1 Kouto
Kamigori
Ako-gun
Hyogo 678-1297
Japan
[2]Department of Bioscience and Biotechnology
Graduate School of Natural Science and Technology
Okayama University
Tsushima-Naka
Okayama 700-8530
Japan

## Summary

**The crystal structures of ADP bound and nucleotide-free forms of molecular chaperone-like diol dehydratase-reactivating factor (DDR) were determined at 2.0 and 3.0 Å, respectively. DDR exists as a dimer of heterodimer $(\alpha\beta)_2$. The α subunit has four domains: ATPase domain, swiveling domain, linker domain, and insert domain. The β subunit, composed of a single domain, has a similar fold to the β subunit of diol dehydratase (DD). The binding of an ADP molecule to the nucleotide binding site of DDR causes a marked conformational change of the ATPase domain of the α subunit, which would weaken the interactions between the DDR α and β subunits and make the displacement of the DDR β subunit by DD through the β subunit possible. The binding of the DD β subunit to the DDR α subunit induces steric repulsion between the DDR α and DD α subunits that would lead to the release of a damaged cofactor from inactivated holoDD.**

## Introduction

Enzymatic radical catalysis is defined as the mechanism of catalysis by which enzymes catalyze chemically difficult reactions by utilizing the high reactivity of radicals (for a review, see Toraya, 2003). Coenzyme $B_{12}$ or adenosylcobalamin (AdoCbl)-dependent enzymes are typical radical enzymes and form an adenosyl radical and Co(II) by homolytic cleavage of the cobalt-carbon (Co-C) bond of the coenzyme (for reviews, see Banerjee, 1999, 2003; Dolphin, 1982; Toraya, 2003). AdoCbl-dependent diol dehydratase (DD) (EC 4.2.1.28) is one of these enzymes and catalyzes the conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to the corresponding aldehydes (Lee and Abeles, 1963; Toraya et al., 1976). Since radical enzymes form a highly reactive catalytic radical in the active site, they tend to undergo mechanism-based inactivation (suicide inactivation) by certain substrates or coenzyme analogs (for a review, see Toraya, 2003). In the case of DD, the dehydration of 1,2-propanediol proceeds linearly with time, but the enzyme undergoes irreversible inactivation by glycerol during catalysis (Bachovchin et al., 1977; Toraya, 2000; Toraya et al., 1976) or $O_2$ inactivation in the absence of substrate (Wagner et al., 1966). Such inactivation is accompanied by the irreversible cleavage of the Co-C bond of the coenzyme (Figure 1A). The resulting damaged cofactor remains tightly bound to apoenzyme, which brings about the inactivation of enzymes. The inactivation of DD by glycerol seemed enigmatic, because glycerol is a growth substrate for the bacteria that produce this enzyme (Abeles, 1966; Toraya, 1999, 2000; Toraya et al., 1978, 1980). Rapid reactivation takes place in situ (in toluenized cells) when ATP and $Mg^{2+}$ are added to the completely inactivated system in the presence of AdoCbl (Honda et al., 1980; Ushio et al., 1982). We found that a proteinaceous factor is responsible for the rapid reactivation of the inactivated holoDD in the presence of AdoCbl, ATP, and $Mg^{2+}$ and named it a DD-reactivating factor (DDR) (Mori et al., 1997a).

We identified two open reading frames in the 3′ flanking region of the DD genes (*pddABC*) of *Klebsiella oxytoca* as the genes encoding DDR and designated them *ddrAB* (corresponding to *pduGH*) (Figure 1B) (Mori et al., 1997b). Recombinant DdrA (α subunit) and DdrB (β subunit) form a tight $\alpha_2\beta_2$ complex that actually functions in vitro as a reactivating factor for glycerol-inactivated holoDD in the presence of AdoCbl, ATP, and $Mg^{2+}$ (Toraya and Mori, 1999). $O_2$-inactivated holoenzyme and the inactive enzyme·cyanocobalamin complex also undergo rapid reactivation and activation, respectively, by DDR under the same conditions. DDR mediates the ATP-dependent exchange of the enzyme bound cyanocobalamin for free adeninylpentylcobalamin, but the reverse is not the case. It was demonstrated that the function of DDR is to release a tightly bound adenine-lacking cobalamin from the enzyme. The apoenzyme formed is reconstitutable into active holoenzyme (Figure 1A) (Mori and Toraya, 1999). Cobalamins containing the adenine ring in the upper axial ligand, such as AdoCbl and adeninylpentylcobalamin, are not released from the enzyme under the conditions. ADP bound and ATP bound DDRs are the high-affinity and low-affinity forms of DDR, respectively. It was established that the reactivation of the inactivated holoenzymes by DDR takes place in two steps: (1) ADP-dependent cobalamin release and (2) ATP-dependent dissociation of the resulting apoenzyme·DDR complex (Figure 1C). ATP plays dual roles, that is, as a precursor of ADP for the first step and as an effector that induces the conformational change of DDR into the low-affinity form for DD. As suggested by sequence homologies (Mori et al., 1997b), *gdrAB* (*dhaB4* and orf2b) of *Klebsiella pneumoniae* (Tobimatsu et al., 1999) and *dhaFG* of *Citrobacter freundii* (Seifert et al., 2001), the orthologs of *gdrAB*, were identified as the genes encoding a glycerol dehydratase-reactivating factor (GDR). GDR reactivates the inactivated glycerol dehydratase by a similar

*Correspondence: toraya@cc.okayama-u.ac.jp

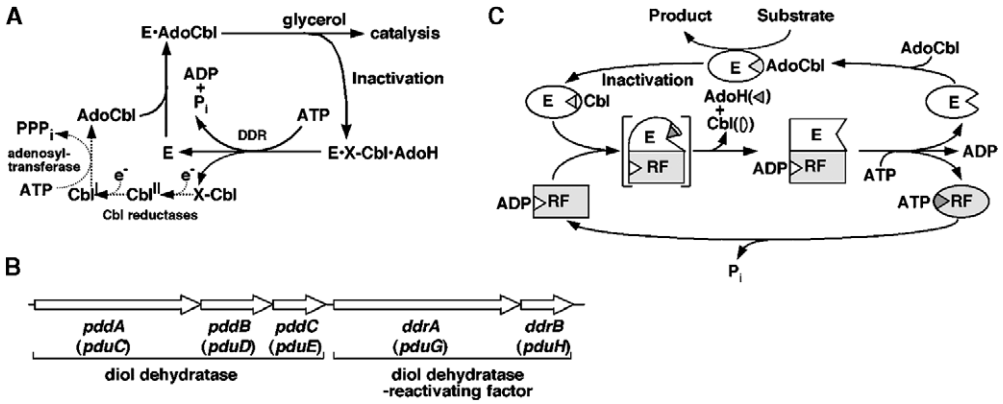


Figure 1. Diol Dehydratase-Reactivating Factor

(A) Reactivation of inactivated holoDD by exchange of a damaged cofactor for intact coenzyme. AdoH, 5′-deoxyadenosine; $Cbl^{II}$, cob(II)alamin; $Cbl^{I}$, cob(I)alamin; X-Cbl, a damaged cofactor. Dotted lines indicate the reactions outside of DD.

(B) Organization of the genes for DD and DDR in the *pdu* operon.

(C) Postulated mechanism of reactivation of inactivated holoDD by DDR. E, apoDD; RF, DDR; AdoH, 5′-deoxyadenosine; Cbl, cobalamin.

mechanism to that of DDR (Kajiura et al., 2001). Very recently, we identified *eutA* of *Escherichia coli* as the gene encoding an ethanolamine ammonia lyase-reactivating factor (Mori et al., 2004). These are a new type of molecular chaperones that are involved in the reactivation of inactivated enzymes (Kajiura et al., 2001; Mori and Toraya, 1999; Mori et al., 2004; Toraya, 2003; Toraya and Mori, 1999). They actually show fragmentary sequence similarities with Hsp70 family molecular chaperones (Mori et al., 1997b, 2004; Tobimatsu et al., 1999). The regions that show similarity constitute the ADP binding site of Hsp70 (Bork et al., 1992; Flaherty et al., 1990), and this finding led us to predict that they may share the actin-like fold. The reactivating factors mediate the release of a tightly bound, damaged cofactor from the inactivated holoenzymes. The damaged cofactor released is back-converted to AdoCbl by reductive adenosylation outside of the enzymes (Figure 1A).

In this paper, we report the X-ray structures of ADP bound and nucleotide-free forms of DDR of *K. oxytoca*. The mechanism of release of a damaged cofactor from inactivated holoDD upon ADP binding to DDR is also discussed here. A preliminary paper on the crystallization of DDR has recently been published (Mori et al., 2005). A paper that reported the X-ray structure of the nucleotide-free form of GDR has been published (Liao et al., 2003b).

## Results and Discussion

### Overall Structure

Both ADP bound and nucleotide-free forms of DDR were crystallized in the $P2_12_12_1$ space group with two $\alpha\beta$ heterodimers in the asymmetric unit. As deduced from the molecular weight determination (Toraya and Mori, 1999), DDR is composed of two $\alpha$ subunits and two $\beta$ subunits that are actually assembled as a dimer of $\alpha\beta$ heterodimer related by a noncrystallographic 2-fold axis (Figure 2A). The interaction between the two $\alpha$ subunits mainly contributes to the dimerization of the heterodimers. The two $\beta$ subunits are separately bound to each of the two $\alpha$ subunits, but there is some interaction between them in the heterotetramer.

### Structure of Each Subunit

The $\alpha$ subunit is a gun-shaped molecule that has four domains, namely, the ATPase domain, the swiveling domain, the linker domain, and the insert domain (Figures 2B and 2C), that are designated after the structure of GDR (Liao et al., 2003b). All of the domains found in the $\alpha$ subunit of GDR are conserved in the structure of the DDR $\alpha$ subunit as well. It should be noted that each domain of the $\alpha$ subunit has two connection sites to its adjacent domain(s): ATPase domain-linker domain at A76-T77 and M370-Q369, ATPase domain-insert domain at Y464-P465 and D511-G510, linker domain-swiveling domain at S96-T97 and T249-K248. This means that each domain, except the ATPase domain, is inserted in its upstream domain, not just linked with a single linkage.

The ATPase domain, located in the grip of the gun, is a core domain, which generates the driving force of reactivating factor activity. The ADP molecule occupies the nucleotide binding site of DDR in a similar manner as that found in the ATPase domain of hsc70. This domain has no direct interaction with the $\beta$ subunit, but it can indirectly influence the interaction of the $\beta$ subunit with the $\alpha$ subunit through the insert domain, which is directly connected to the ATPase domain, and the swiveling domain of the neighboring $\alpha$ subunit. The linker domain is a $\beta$ strand-rich domain connecting the swiveling domain and the ATPase domain. Three $\beta$ strands, $\beta$5, $\beta$17, and $\beta$18, that form the backbone of the linker domain and the barrel of the gun, are almost parallel or antiparallel to the $\alpha$10 and $\alpha$12 helices in the ATPase domain (Figures 2B and 3). These three $\beta$ strands of the linker domain form tight interactions with the ATPase domain, mainly through these two helices. This domain is the only domain that has no van der Waals contact to the other subunits. The role of this domain is probably to connect the ATPase and swiveling domains and to hold the swiveling domain at the proper position.

The insert domain, the trigger of the gun, is located between the $\alpha$11 and $\alpha$12 helices of the ATPase domain and can be regarded as a part of the ATPase domain. The insert domain is the simplest in this protein and

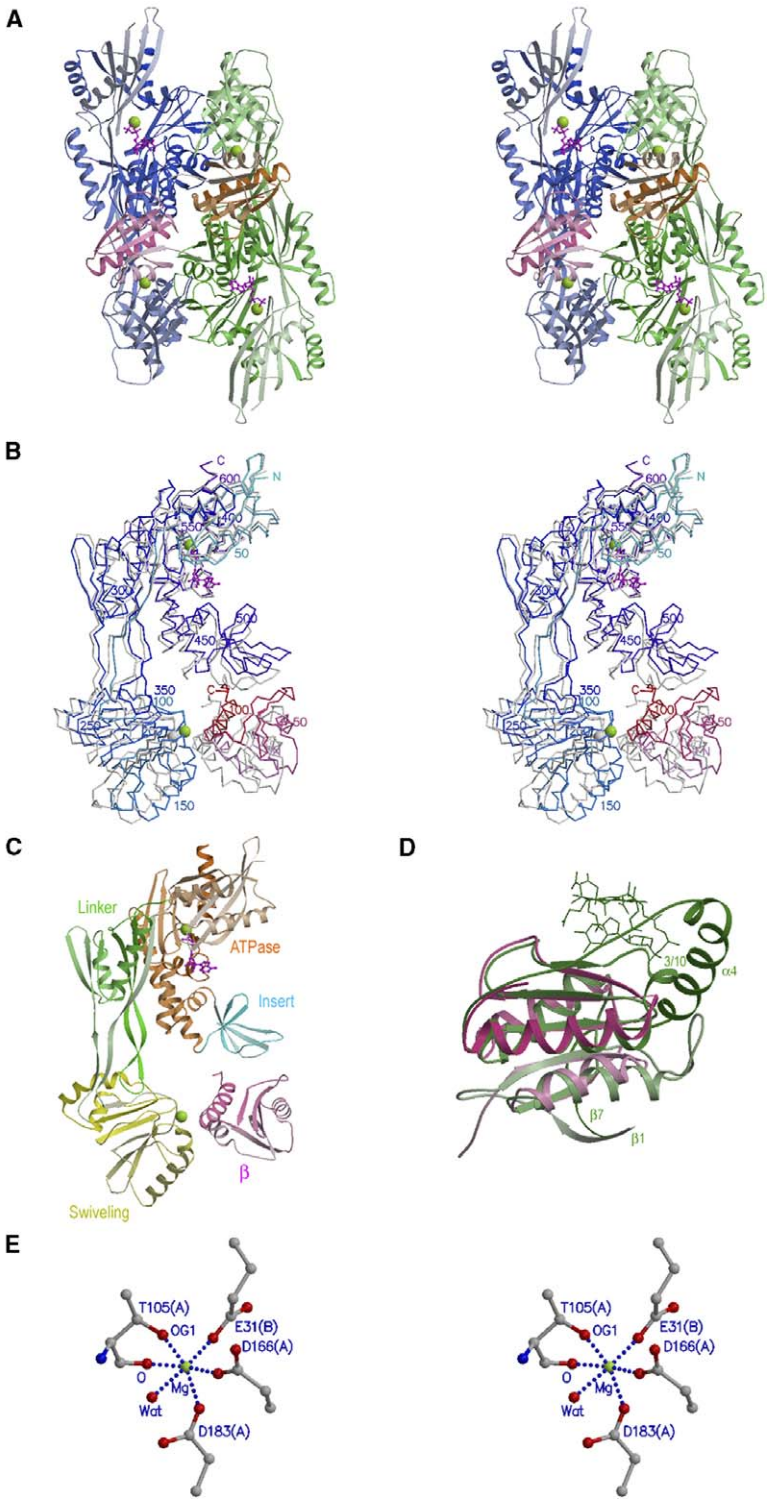


Figure 2. The Structure of DDR in the ADP Bound Form

(A) Ribbon drawing of the DDR. The color codes for each subunit: blue, α subunit (A chain); pink, β subunit (B chain); green, α subunit (C chain); orange, β subunit (D chain). The colors gradually deepen as residue numbers increase. The bound ADP molecule and divalent cations are shown in magenta ball-and-stick and green sphere models.

(B) The Cα trace of the DDR αβ heterodimer. Colors ranging from cyan to purple are used for the α subunit, and colors ranging from pink to red are used for the β subunit. The stick model shown in light gray indicates the αβ dimer in the nucleotide-free form.

(C) Domain structure of the DDR αβ heterodimer.

(D) Structural comparison between the DDR β subunit (pink) and the DD β subunit (dark green). The cobalamin molecule of DD is shown as a stick model. The labels α4, β1, β7, and $3_{10}$ indicate α4-DDβ, β1-DDβ, β7-DDβ, and $3_{10}$-DDβ, respectively.

(E) The metal ion binding site between the α and β subunits in the ADP bound form of DDR. Amino acids involved in metal binding are shown in ball-and-stick models. These figures were generated by using the programs MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt and Bacon, 1997).

contains a five-stranded antiparallel β sheet. The insert domain bridges the gap between the ATPase domain and the β subunits in the same and neighboring dimers. The swiveling domain, inserted between S96 and T249 of the linker domain and located at the muzzle of the gun, has no direct interaction with the ATPase domain of the same α subunit, unlike the insert and linker domains. In the $(\alpha\beta)_2$ heterotetramer, however, the swiveling domain structurally connects the β subunit and the ATPase domain of the other α subunit. The swiveling domain has van der Waals contact with the C-terminal end of the α1 helix of the β subunit.

The β subunit displays a Rossmann-like fold with five β strands and three α helices (Figure 3). As suggested from the sequence similarity with the β subunits of DD (Mori et al., 1997b) and glycerol dehydratase (Tobimatsu

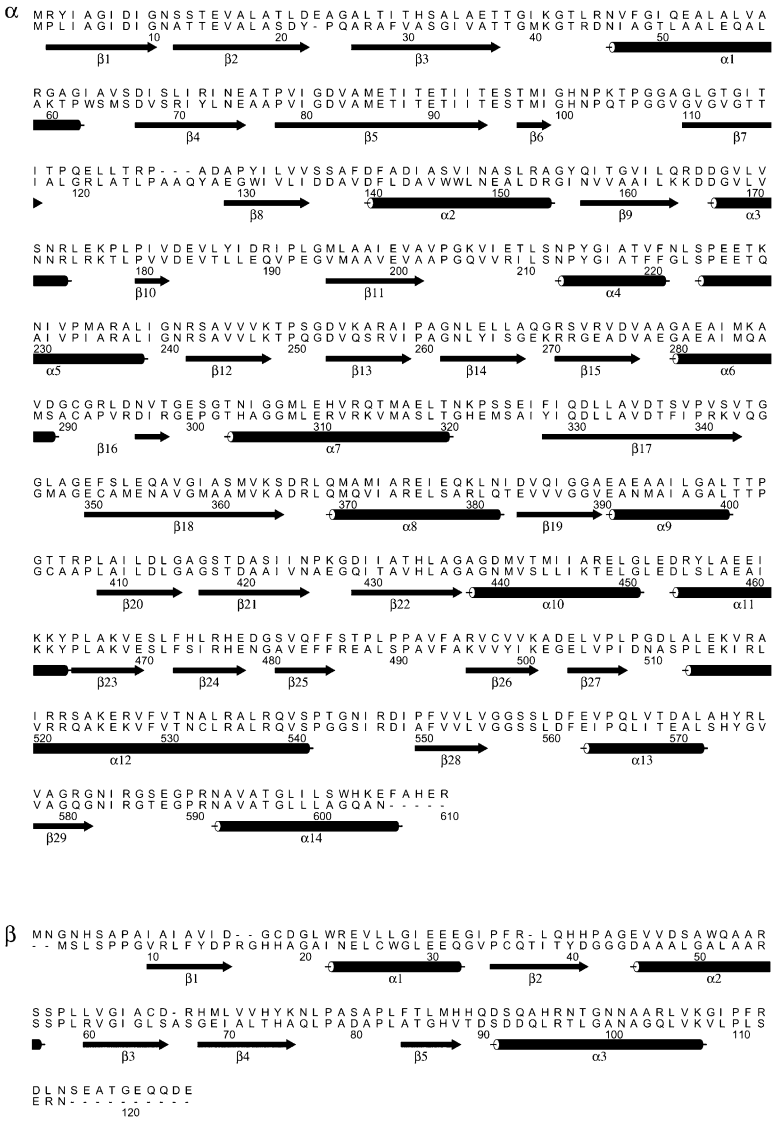

Figure 3. Alignment of the Amino Acid Sequences of DDR and GDR

The secondary structural elements based on those of DDR are marked above the sequence alignment. The α and β subunits are shown sequentially. This figure was drawn with ALSCRIPT (Barton, 1993).

et al., 1999), the β subunit has a similar fold to the GDR β subunit (Liao et al., 2003b) and to the β subunits of DD (Shibata et al., 1999) or glycerol dehydratase (Liao et al., 2003a; Yamanishi et al., 2002), except that the first (β1-DDβ) and the last (β7-DDβ) β strands, the $3_{10}$ helix ($3_{10}$-DDβ), and the fourth α helix (α4-DDβ) of the DD β subunit are lacking in the DDR β subunit (Figure 2D). Both the $3_{10}$-DDβ and the α4-DDβ regions contain important residues for cobalamin binding to DD. Moreover, other residues involved in cobalamin binding are not conserved in DDR. This would indicate that the binding affinity of the DDR β subunit to cobalamin is much lower or almost lost.

## Subunit Interactions

The domains of the α subunit located in the interface between the α and β subunits and thus suggested to be involved in the interaction with the β subunit are the swiveling domain of the same αβ dimer and the insert domains of both α subunits. Salt bridges and hydrogen bonds are predominant on the interface between the β subunit and the swiveling domain, whereas hydrophobic interactions play a main role in the interaction between the β subunit and the insert domains of both α subunits.

It should be noted that a metal ion found on the interface between the α and β subunits seems to play an important role in the formation of the αβ dimer, although hydrophobic force is mainly responsible for the interactions between them. Six ligands, OD1(Dα166), OD1 (Dα183), O(Tα105), OG1(Tα105), OE1(Eβ31), and a water molecule, coordinate to the metal ion (Figure 2E). All 4 amino acid residues are completely conserved in both DDR and GDR. The coordinations of these residues to the metal ion are maintained in DDR no matter whether ADP is bound or not. In the ADP bound form of DDR, however, the temperature factor of the metal ion became significantly higher than the averaged value of its ligating atoms by approximately 11 $Å^2$ (38.3 $Å^2$ versus 26.7 $Å^2$) when refined as a $Ca^{2+}$ ion, whereas it became comparable (27.5 $Å^2$ versus 26.7 $Å^2$) when refined as a $Mg^{2+}$ ion. The averaged bond distance between the carboxylates and the metal ion is 2.20 Å, comparable to the typical value for the $Mg^{2+}$ ion (2.26 Å) and

significantly shorter than the value for the $Ca^{2+}$ ion (2.36 Å) (Harding, 2001). The distances between the other ligating atoms and the metal ion are shorter than the values for the $Ca^{2+}$ ion. These lines of evidence indicate that the $Mg^{2+}$ ion is a more suitable metal ion than the $Ca^{2+}$ ion in the present structure of the ADP bound form of DDR, although they are indirect proofs. This metal ion has been reported to be a $Ca^{2+}$ ion in the nucleotide-free form of GDR (Liao et al., 2003b). In the case of the nucleotide-free form, we assigned a $Ca^{2+}$ ion to the metal ion in this site, since the temperature factor of the ion was comparable to those of the ligating atoms when refined as a $Ca^{2+}$ ion. It was thus suggested that the $Ca^{2+}$ ion bound at this site of the nucleotide-free form of DDR is substituted by a $Mg^{2+}$ ion in the ADP bound form when DDR is incubated with ADP and $Mg^{2+}$. Since ATP and $Mg^{2+}$ are absolutely required for the reactivation, it is likely that $Mg^{2+}$ is a functional divalent cation. It might be possible that a $Mg^{2+}$ ion occupies this site in vivo and is replaced by a $Ca^{2+}$ ion in the purification of DDR through hydroxyapatite column chromatography (Toraya and Mori, 1999).

### Binding of ADP and $Mg^{2+}$

As expected from the functional and fragmentary sequence similarities with Hsp70 family molecular chaperones, an ADP molecule is bound at the nucleotide binding site of DDR in a similar manner to that of hsc70 (Flaherty et al., 1990). The oxygen atoms of α and β phosphates form hydrogen bonds to amide nitrogen atoms of the backbone (Figure 4A), similar to what happens with hsc70. Also, two hydrogen bonds with the ribose moiety, OE2(Eα459)-O2′(ribose) and NZ(Kα462)-O2′(ribose), are conserved in hsc70. The hydrogen bond OG1(Sα13)-O2A(phosphate) and the salt bridge NH1(Rα591)-O2A(phosphate) are absent in hsc70. Another $Mg^{2+}$ ion is also found at the nucleotide binding site. This $Mg^{2+}$ ion is octahedrally coordinated by an oxygen atom of the β phosphate group and five water molecules.

Upon ADP binding, the largest conformational change occurs in the ATPase domain, that is, the distance between the two domains composing the ATPase domain becomes closer, whereas the linker domain shows the smallest deviation (Figures 2B and 4B). This means that the movement of the swiveling domain is brought about through the movement of another part of the molecule. The ATPase domain has direct contact with the swiveling domain of the other α subunit. For example, the direction of the movement of the swiveling domain in the C chain is almost the same as that of the ATPase domain in the neighboring α subunit (A chain) and that of the adjacent β subunit (D chain). The linker domain, covalently linked to the ATPase domain, shows smaller conformational change than the swiveling domain. This means that the linker domain acts as a flat spring or a hinge for the domain movement of the swiveling domain.

Another important change is the movement of the insert domain. Other than the largest conformational change in the ATPase domain, the second largest conformational change is found in the insert domain. The insert domain is directly linked to the α11 helix of the α subunit, whose Eα459 and Kα462 form hydrogen bonds to the 2′-OH group of the ribose moiety of ADP, as described above. It is worth noting that Kα463, another residue in the α11 helix of the α subunit, forms a charge-transfer cation-π interaction (Ma and Dougherty, 1997) (Figure 4A). The corresponding residue in hsc70 is arginine, indicating that an amino acid residue with a positive charge at this position is important for the binding of the nucleotide base moiety. These interactions pull the α11 helix toward the base of the molecule, resulting in the movement of the insert domain. The structural changes of the insert domain and the adjacent parts of the ATPase domain upon ADP binding seem to be mainly induced due to these two hydrogen bonds with the ribose moiety, whereas the conformational change of the rest of the domain is caused by the binding of the pyrophosphate group of ADP.

The total contact area between the DDR α and β subunits is 1,209 and 1,391 $Å^2$ in the AB and CD dimers, respectively, in the ADP bound form and 1,154 and 1,251 $Å^2$ in the AB and CD dimers, respectively, in the nucleotide-free form. However, the contact area between the DDR β subunit and the insert domain of the same α subunit was 325 and 375 $Å^2$ in the AB and CD dimers, respectively, in the ADP bound form and 362 and 397 $Å^2$ in the AB and CD dimers, respectively, in the nucleotide-free form. Thus, the interaction of the β subunit with the insert domain in the same dimer may become slightly weakened by ADP binding to the DDR α subunit. This would be because the ATPase domain takes a closed conformation upon ADP binding (Figure 2B). In contrast, the β subunit moves with the swiveling domain, because the amino acid residues from both subunits coordinate to the $Mg^{2+}$ ion. It should be noted that both the ADP bound and nucleotide-free forms of DDR form a tight complex with apoDD (Mori and Toraya, 1999). This may be the reason why the total contact areas are not so much affected by the binding of ADP. For full understanding of the nucleotide-switching mechanism of DDR, we have to await the crystal structure determination of DDR in complex with ATP or its nonhydrolyzable analog.

### Possible Interactions of DDR with DD

As shown in Figure 2D, the fold of the DDR β subunit is very similar to that of the DD β subunit, which has a fold similar to the β subunit of glycerol dehydratase, as well. In addition, it was suggested that the interaction between the α and β subunits of DDR might be weakened, at least partly, by ADP binding to the nucleotide binding site in the DDR α subunit. Therefore, the displacement of the DDR β subunit by DD through the β subunit would be possible. Two kinds of displacement models could be considered. If the α and β subunits of DDR are abbreviated as $\alpha_R$ and $\beta_R$, respectively, and the α, β, and γ subunits of DD are abbreviated as $\alpha_D$, $\beta_D$, and $\gamma_D$, respectively, for simplicity, one model involves the formation of the hypothetical $(\alpha_R \cdot \beta_D\alpha_D\gamma_D)_2$ complex, and the other model involves the formation of the hypothetical $(\alpha_R\beta_R \cdot \alpha_D\gamma_D)_2$ complex. The subunit exchange model in which $(\alpha_R \cdot \beta_D)_2$ or $(\beta_R \cdot \alpha_D\gamma_D)_2$ are formed seems to be less likely, because the displacement of the DDR β or DD β subunit by entire DD or DDR molecules would be energetically more favorable than that by the corresponding subunit itself. For the formation of $(\alpha_R \cdot \beta_D\ \alpha_D\gamma_D)_2$ and $(\alpha_R\beta_R \cdot \alpha_D\gamma_D)_2$ complexes, dissociation of αβ in DDR and αβ in DD, respectively, is required. The

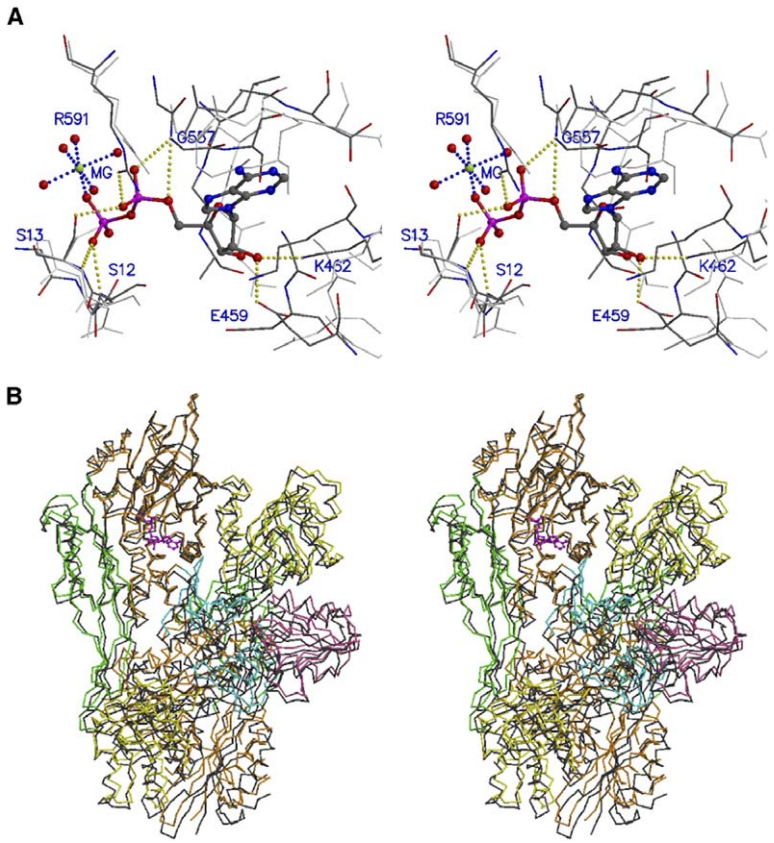


Figure 4. Comparison of the Structure between the ADP Bound and Nucleotide-free Forms

(A) The ADP binding site. The stick models colored in the by-atom coding and in light gray indicate the ADP bound and nucleotide-free forms, respectively. Yellow and blue dotted lines are hydrogen bonds and coordination bonds, respectively.

(B) The overall view. The ADP bound form is colored as in Figure 2C. The nucleotide-free form (gray) is superimposed on that of the ADP bound form. The B chain is not shown for simplicity.

ADP molecule binds to the DDR $\alpha$ subunit. Therefore, the formation of the former complex seems to be more likely than that of the latter complex, although direct biochemical or structural evidence for this is not yet available. Liao et al. (2003b) also postulated a similar GD·GDR complex in their "subunit swap" model.

The docking model of the $(\alpha_R \cdot \beta_D \alpha_D \gamma_D)_2$ complex was constructed from the structures of DDR and DD by superimposing the structure of the DDR $\beta$ subunit onto that of the DD $\beta$ subunit (Figure 5A). The modeled structure indicates that there is marked steric repulsion between the insert domain of the ADP bound DDR $\alpha$ subunit and the $\alpha$22 helix of the DD $\alpha$ subunit and between the $\alpha$1 helix (mainly E52) of the DDR $\alpha$ subunit and the DD $\alpha\gamma$ subunits. The parts of the DD $\alpha$ subunit pushed by DDR $\alpha$ are marked in red in Figure 5. It should be noted that the two dimers are not completely symmetrical when the DD $\beta$ subunit is superimposed onto the B or D chain of DDR. As shown in Figure 5B, there is main repulsion between the insert domain of the ADP bound DDR $\alpha$ chain and the $\alpha$22 helix of the DD $\alpha$ subunit. In the C chain, however, the repulsion at this region is less than in the A chain, but the repulsion between the $\alpha$1 helix of the DDR C chain and the DD $\alpha\gamma$ subunits is more than in the A chain. The numbers of atoms that come closer than 3.5 Å in the modeled structures of $(A \cdot \beta_D \alpha_D \gamma_D)_2$ and $(C \cdot \beta_D \alpha_D \gamma_D)_2$ complexes are 42 and 79, respectively, in the ADP bound form and 60 and 65, respectively, in the nucleotide-free form. It is thus suggested that ADP binding to DDR increases the repulsion between the DDR C chain and the DD $\alpha$ subunit in the $(C \cdot \beta_D \alpha_D \gamma_D)_2$ complex, whereas it decreases the repulsion between the DDR A chain and the DD $\alpha$ subunit in the $(A \cdot \beta_D \alpha_D \gamma_D)_2$ complex. Thus, the amino acid side chains that come closer than the van der Waals contact in the modeled structures would push aside each other, which could bring about the tilting of the DD $\alpha$ subunit with respect to the DD $\beta$ subunit. The positions of these residues in the insert domain of the $\alpha$ subunit move away by about 6 Å in the nucleotide-free form of DDR, and, thus, the steric repulsion seems to be much less or negligible in the absence of ADP. There are no direct interactions between DD and either the ATPase or linker domains in the modeled structure of the $(\alpha_R \cdot \beta_D \alpha_D \gamma_D)_2$ complex (Figure 5A). The swiveling domain has no direct interaction with DD, except for that with the DD $\beta$ subunit.

### Proposed Mechanism for the Release of a Damaged Cofactor from DD upon ADP Binding to DDR

From the X-ray structures of the ADP bound and nucleotide-free forms of DDR as well as the above-mentioned modeling study, we propose here the mechanism shown in Figure 6 for the reactivation of DD by DDR. Binding of ADP to the ATPase domain of DDR induces the marked conformational change of its $\alpha$ subunits. This seems to be transmitted to the $\beta$ subunits through the swiveling domain of the neighboring $\alpha$ subunit and the insert domain of the same $\alpha$ subunit. As a result, the interaction between the $\alpha$ and $\beta$ subunits could be weakened. Since the fold of the DDR $\beta$ subunit is very similar to that of the DD $\beta$ subunit, it would be reasonable to assume that the displacement of the DDR $\beta$ subunit by DD through the $\beta$ subunit becomes possible. The modeling study of the

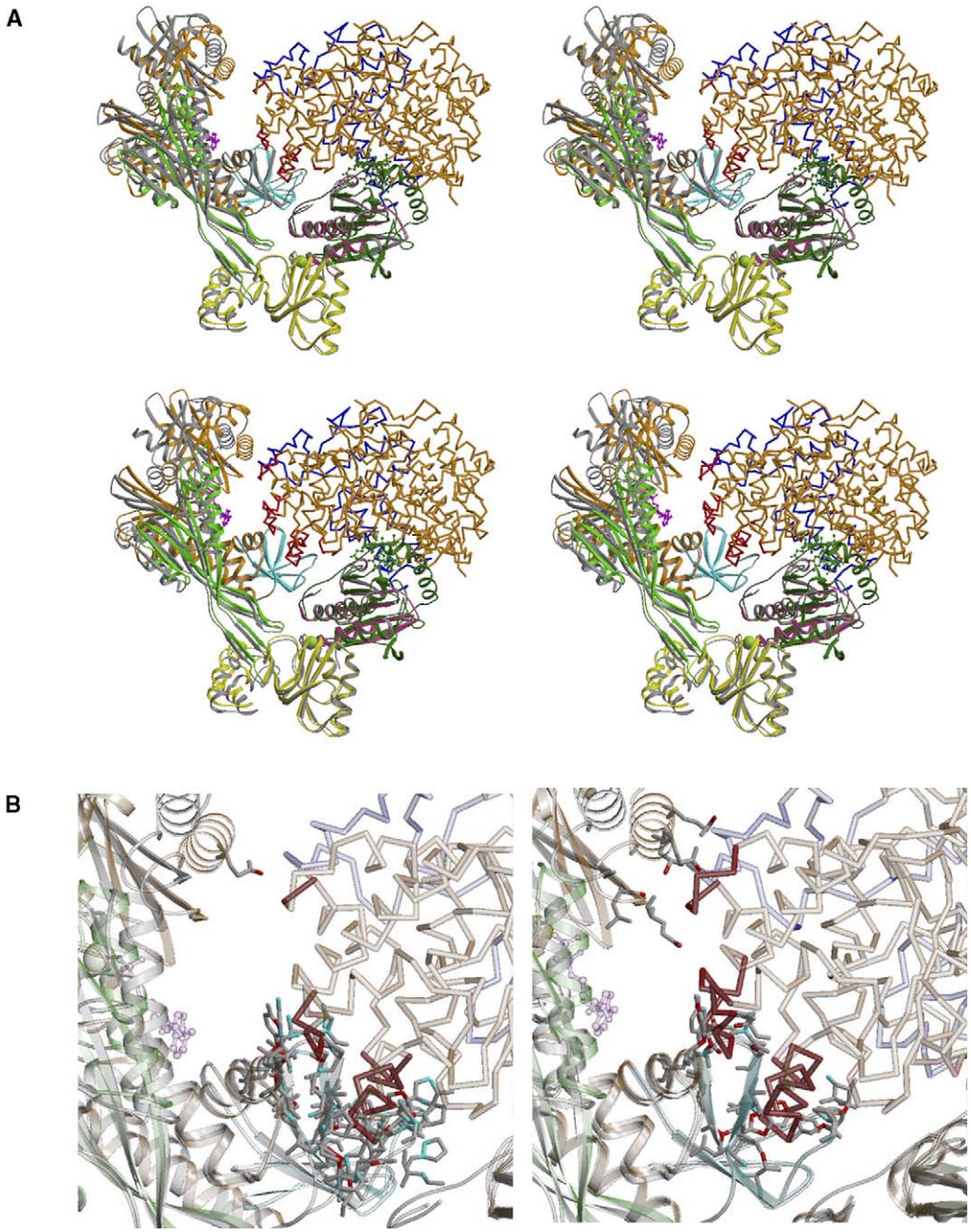


Figure 5. The Docking Model of the DD-DDR Complex

(A) The overall view. The DDR B and D chains in the nucleotide-free form and the DD β subunit are superimposed on B (upper panel) and D (lower panel) chains in the ADP bound form, respectively. The DD α and γ subunits are shown in the orange and blue Cα-trace models, and the residues of DD contacting the DDR α subunit are highlighted in red. The color codes of the other components are the same as in Figures 2D and 4B.
(B) The close-up view of the contacting region. Stick models are displayed for the residues containing the atoms of DDR contacting or overlapping DD. These atoms are shown in red for the ADP bound form and in blue for the nucleotide-free form. The contact regions of the A·$\beta_D\alpha_D\gamma_D$ and C·$\beta_D\alpha_D\gamma_D$ complexes are shown on the left and right, respectively.

(A·$\beta_D\alpha_D\gamma_D$)$_2$ and (C·$\beta_D\alpha_D\gamma_D$)$_2$ complexes suggests that the binding of the DD β subunit to the ADP bound form of the DDR α subunit (A and C chains) induces the steric repulsion between the insert domain of the DDR C chain and the DD α subunit, that is, the insert domain and the α1 helix of the ADP bound C chain pushes aside the α22 helix and other parts of the DD α subunit. This would cause the tilting of the DD α subunit with respect to the DD β subunit and thus lead to the release of the damaged cofactor, an adenine-lacking cobalamin, from DD, because cobalamin is bound between the α and β subunits of the enzyme.

One of the structures reported here is the first, to our knowledge, structure of the reactivating factors for AdoCbl-dependent enzymes in the ADP bound form. From the structural similarity with Hsp70 family

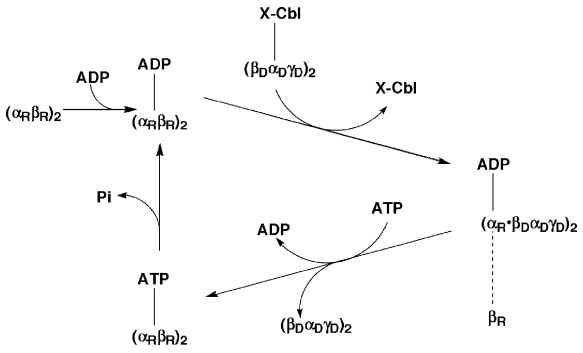


Figure 6. Proposed Mechanism of the Reactivation of Inactivated holoDD by DDR

X-Cbl, a damaged cofactor. $\alpha_R$ and $\beta_R$, $\alpha$ and $\beta$ subunits of DDR; $\alpha_D$, $\beta_D$, and $\gamma_D$, $\alpha$, $\beta$, and $\gamma$ subunits of DD. $(\alpha_R{\cdot}\beta_D\alpha_D\gamma_D)_2$ represents one of the hypothetical forms of the DD·DDR complex, and other forms of the complex can also be considered.

molecular chaperones, it was suggested that DDR, GDR, and Hsp70 molecular chaperones are evolved from a common ancestor protein (Toraya, 2003). The structures and mechanisms of their nucleotide (ADP/ATP) switches might be conserved in their molecular evolution.

## Experimental Procedures

### Expression and Purification of DDR

Wild-type and selenomethionine-substituted DDRs of *K. oxytoca* were overexpressed in methionine-auxotrophic *E. coli* B834 (Novagen) harboring expression plasmid pUSI2ENd(6/5b) (Toraya and Mori, 1999) that were grown in the presence of methionine and selenomethionine, respectively (Mori et al., 2005). DDRs were purified to homogeneity according to the method described previously (Toraya and Mori, 1999).

### Crystallization

Both wild-type and selenomethionine-substituted DDRs were crystallized in the nucleotide-free and ADP bound forms under similar conditions (Mori et al., 2005). Purified DDRs (20–40 mg/ml in 10 mM potassium phosphate buffer [pH 8.0]) were incubated at 30ºC for 30 min with either 50 mM 2-mercaptoethanol (for the ADP bound form) or 10 mM dithiothreitol (for the nucleotide-free form). To 2-mercaptoethanol-treated DDR were added 2 mM ADP and 2 mM $MgCl_2$, and the mixtures were incubated at 20ºC for 20 min. Crystals were grown by the sandwich-drop vapor-diffusion method at 4ºC against 0.5 ml reservoir solution containing 15% PEG 6000, 90 mM ammonium sulfate, 12 mM Tris-HCl (pH 8.0), 20% PEG 400, and either 50 mM 2-mercaptoethanol (for the ADP bound form) or 10 mM dithiothreitol (for the nucleotide-free form). The crystal-growth droplet was composed of 30 μl DDR solution and 30 μl reservoir solution.

### Data Collection

All of the X-ray diffraction data sets were measured at 100 K. The data sets of the ADP bound and nucleotide-free forms of wild-type DDR (Mori et al., 2005) and the MAD data sets of the ADP bound form were collected at the BL41XU and BL38B1 beamlines, SPring-8, Japan, respectively. Three data sets close to the K absorption edge of selenium were obtained. A total of 180 images with 1° oscillation were recorded for each data set by using the ADSC CCD detector system and were processed and scaled by using *HKL*2000 (Otwinowski and Minor, 1997). The auto-indexing indicated that the crystal belongs to one of the orthorhombic space groups, and the reflection conditions suggested space group $P2_12_12_1$. The Matthews volumes, $V_M$ (Matthews, 1968), are 3.17 and 3.33 $Å^3Da^{-1}$ for the ADP bound and nucleotide-free forms of wild-type DDR, respectively, assuming one $(\alpha\beta)_2$ heterotetramer (two $\alpha\beta$ heterodimers) per asymmetric unit (Mori et al., 2005). These correspond to the solvent contents of 61.2% and 63.1%, respectively. The summary of data collection and phasing statistics is listed in Table 1.

Table 1. Data Collection and Refinement Statistics

| | ADP Bound Form | | | | Nucleotide-free Form |
|---|---|---|---|---|---|
| | Native | Peak | Edge | Remote | Native |
| **Data Collection and Phasing Statistics** | | | | | |
| Space group | $P2_12_12_1$ | $P2_12_12_1$ | | | $P2_12_12_1$ |
| Unit cell parameters (Å) | a = 83.26, b = 84.60, c = 280.09 | a = 83.57, b = 85.10, c = 281.06 | | | a = 81.92, b = 85.37, c = 296.99 |
| Wavelength (Å) | 1.00000 | 0.97904 | 0.97922 | 0.97100 | 1.00000 |
| Resolution range (Å) | 50–2.00 (2.07–2.00) | 50–2.60 (2.69–2.60) | 50–2.60 (2.69–2.60) | 50–2.60 (2.69–2.60) | 30–3.00 (3.11–3.00) |
| Measured reflections | 925,259 | 435,335 | 436,384 | 437,574 | 287,550 |
| Unique reflections | 133,910 | 62,041 | 62,160 | 62,207 | 41,979 |
| Completeness (%) | 99.9 (99.8) | 99.0 (90.6) | 99.0 (90.6) | 99.4 (94.4) | 97.7 (93.3) |
| $R_{merge}$ (%) | 8.7 (59.8) | 9.8 (38.1) | 8.4 (38.7) | 8.8 (40.0) | 7.9 (60.6) |
| Multiplicity | 6.9 (5.0) | 7.0 | 7.0 | 7.0 | 6.8 |
| $I/\sigma(I)$ | 11.2 | 16.6 | 16.0 | 16.0 | 8.9 |
| **Refinement Statistics** | | | | | |
| Resolution range (Å) | 50–2.00 (2.07–2.00) | | | | 50–3.00 (3.11–3.00) |
| $R_{work}$ (%) | 19.8 (28.1) | | | | 23.8 (46.0) |
| $R_{free}$ (%) | 23.2 (31.5) | | | | 31.3 (51.1) |
| Number of protein atoms | 10,598 | | | | 10,633 |
| Number of heteroatoms | 68 | | | | 27 |
| Number of water molecules | 806 | | | | 33 |
| Rms deviations from ideal values | | | | | |
| Bond lengths (Å) | 0.005 | | | | 0.007 |
| Bond angles (º) | 0.9 | | | | 1.1 |

### Structural Determination and Refinement

Initial phases for the ADP bound form were determined by the MAD method by using the program SHARP (de La Fortelle and Bricogne, 1997). Selenium sites were identified by the program SnB (Weeks and Miller, 1999). A partial model was automatically built by using the program ARP/wARP (Perrakis et al., 1999), followed by manual building for the missing residues by the program XFIT (McRee, 1993). The initial model was then used for positional and B factor refinement against the native data set of the ADP bound form by the program CNS (Brunger et al., 1998), followed by several cycles of manual model rebuilding by XFIT and refinement to give an R factor of 19.8% (Table 1).

The structure of the ADP-free form was solved by the molecular replacement method by using the ADP bound form as an initial model. The crossrotation and translation functions calculated by the program CNS clearly indicated the correct rotation and translation parameters. Refinement for this form was carried out by the similar method applied for the ADP bound form. The final refinement statistics are given in Table 1.

### Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research ([B] 13480195 and 17370038 and Priority Areas 753) from the Japan Society for Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Grant of Natural Sciences Research Assistance from the Asahi Glass Foundation, Tokyo, Japan to T.T. The synchrotron radiation experiments were performed at the SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) (Proposal Nos. 2004A0653-NL1-np-P3k and 2004B0810-NL1-np-P3k). We thank Dr. Hasegawa, Dr. Sakai, Dr. Kawamoto, and Dr. Shimizu for their kind help in data collection at the BL38B1 and BL41XU beamlines, SPring-8, Japan. We thank Ms. Yukiko Kurimoto for her assistance in manuscript preparation.

Received: June 25, 2005
Revised: August 4, 2005
Accepted: August 4, 2005
Published: December 13, 2005

### References

Abeles, R.H. (1966). Dehydrations requiring vitamin $B_{12}$ coenzyme. Methods Enzymol. *123*, 686–689.

Bachovchin, W.W., Eagar, R.G., Jr., Moore, K.W., and Richards, J.H. (1977). Mechanism of action of adenosylcobalamin: glycerol and other substrate analogues as substrates and inactivators for propanediol dehydratase - kinetics, stereospecificity, and mechanism. Biochemistry *16*, 1082–1092.

Banerjee, R., ed. (1999). Chemistry and Biochemistry of $B_{12}$ (New York: John Wiley & Sons).

Banerjee, R. (2003). Radical carbon skeleton rearrangements: catalysis by coenzyme B12-dependent mutases. Chem. Rev. *103*, 2083–2094.

Barton, G.J. (1993). ALSCRIPT - A tool to format multiple sequence alignments. Protein Eng. *6*, 37–40.

Bork, P., Sander, C., and Valencia, A. (1992). An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. Proc. Natl. Acad. Sci. USA *9*, 7290–7294.

Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. (1998). Crystallography and NMR system (CNS): a new software system for macromolecular structure determination. Acta Crystallogr. *D54*, 905–921.

de La Fortelle, E., and Bricogne, G. (1997). Maximum-likelihood heavy-atom parameter refinement for the multiple isomorphous replacement and multiwavelength anomalous diffraction methods. Methods Enzymol. *276*, 472–494.

Dolphin, D., ed. (1982). $B_{12}$, Volume 2 (New York: John Wiley & Sons).

Flaherty, K.M., DeLuca-Flaherty, C., and McKay, D.B. (1990). Three-dimensional structure of the ATPase fragment of a 70 K heat-shock cognate protein. Nature *346*, 623–628.

Harding, M.M. (2001). Geometry of metal-ligand interactions in proteins. Acta Crystallogr. *D57*, 401–411.

Honda, S., Toraya, T., and Fukui, S. (1980). In situ reactivation of glycerol-inactivated coenzyme B12-dependent enzymes, glycerol dehydratase and diol dehydratase. J. Bacteriol. *143*, 1458–1465.

Kajiura, H., Mori, K., Tobimatsu, T., and Toraya, T. (2001). Characterization and mechanism of action of a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. J. Biol. Chem. *276*, 36514–36519.

Kraulis, J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Crystallogr. *24*, 946–950.

Lee, H.A., Jr., and Abeles, R.H. (1963). Purification and properties of dioldehydrase, an enzyme requiring a cobamide coenzyme. J. Biol. Chem. *238*, 2367–2373.

Liao, D.-I., Dotson, G., Turner, I., Jr., Reiss, L., and Emptage, M. (2003a). Crystal structure of substrate free form of glycerol dehydratase. J. Inorg. Biochem. *93*, 84–91.

Liao, D.-I., Reiss, L., Turner, I., Jr., and Dotson, G. (2003b). Structure of glycerol dehydratase reactivase: a new type of molecular chaperone. Structure *11*, 109–119.

Ma, J.C., and Dougherty, D.A. (1997). The cation-$\pi$ interaction. Chem. Rev. *97*, 1303–1324.

Matthews, B.W. (1968). Solvent content of protein crystals. J. Mol. Biol. *33*, 491–497.

McRee, D.E. (1993). Practical Protein Crystallography (San Diego, CA: Academic Press).

Merritt, E.A., and Bacon, D.J. (1997). Raster3D: photorealistic molecular graphics. Methods Enzymol. *277*, 505–524.

Mori, K., and Toraya, T. (1999). Mechanism of reactivation of coenzyme B12-dependent diol dehydratase by a molecular chaperone-like reactivating factor. Biochemistry *38*, 13170–13178.

Mori, K., Tobimatsu, T., and Toraya, T. (1997a). A protein factor is essential for in situ reactivation of glycerol-inactivated adenosylcobalamin-dependent diol dehydratase. Biosci. Biotechnol. Biochem. *61*, 1729–1733.

Mori, K., Tobimatsu, T., Hara, T., and Toraya, T. (1997b). Characterization, sequencing, and expression of the genes encoding a reactivating factor for glycerol-inactivated adenosylcobalamin-dependent diol dehydratase. J. Biol. Chem. *272*, 32034–32041.

Mori, K., Bando, R., Hieda, N., and Toraya, T. (2004). Identification of a reactivating factor for adenosylcobalamin-dependent ethanolamine ammonia lyase. J. Bacteriol. *186*, 6845–6854.

Mori, K., Hieda, N., Yamanishi, Y., Shibata, N., and Toraya, T. (2005). Crystallization and preliminary X-ray analysis of molecular chaperone-like diol dehydratase-reactivating factor in ADP-bound and nucleotide-free forms. Acta Crystallogr. *F 61*, 603–605.

Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. *276*, 307–326.

Perrakis, A., Morris, R., and Lamzin, V.S. (1999). Automated protein model building combined with iterative structure refinement. Nat. Struct. Biol. *6*, 458–463.

Seifert, C., Bowien, S., Gottschalk, G., and Daniel, R. (2001). Identification and expression of the genes and purification and characterization of the gene products involved in reactivation of coenzyme B12-dependent glycerol dehydratase of *Citrobacter freundii*. Eur. J. Biochem. *268*, 2369–2378.

Shibata, N., Masuda, J., Tobimatsu, T., Toraya, T., Suto, K., Morimoto, Y., and Yasuoka, N. (1999). A new mode of $B_{12}$ binding and the direct participation of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. Structure *7*, 997–1008.

Tobimatsu, T., Kajiura, H., Yunoki, M., Azuma, M., and Toraya, T. (1999). Identification and expression of the genes encoding a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. J. Bacteriol. *181*, 4110–4113.

Toraya, T. (1999). Diol dehydratase and glycerol dehydratase. In Chemistry and Biochemistry of $B_{12}$, R. Banerjee, ed. (New York: John Wiley & Sons), pp. 783–809.

Toraya, T. (2000). Radical catalysis of $B_{12}$-enzymes: structure, mechanism, inactivation, and reactivation of diol and glycerol dehydratases. Cell. Mol. Life Sci. *57*, 106–127.

Toraya, T. (2003). Radical catalysis in coenzyme $B_{12}$-dependent isomerization (eliminating) reactions. Chem. Rev. *103*, 2095–2127.

Toraya, T., and Mori, K. (1999). A reactivating factor for coenzyme $B_{12}$-dependent diol dehydratase. J. Biol. Chem. *274*, 3372–3377.

Toraya, T., Shirakashi, T., Kosuga, T., and Fukui, S. (1976). Substrate specificity of coenzyme $B_{12}$-dependent diol dehydrase: glycerol as both a good substrate and a potent inactivator. Biochem. Biophys. Res. Commun. *69*, 475–480.

Toraya, T., Honda, S., Kuno, S., and Fukui, S. (1978). Coenzyme $B_{12}$-dependent diol dehydratase: regulation of apoenzyme synthesis in *Klebsiella pneumoniae* (*Aerobacter aerogenes*) ATCC 8724. J. Bacteriol. *135*, 726–729.

Toraya, T., Kuno, S., and Fukui, S. (1980). Distribution of coenzyme $B_{12}$-dependent diol dehydratase and glycerol dehydratase in selected genera of *Enterobacteriaceae* and *Propionibacteriaceae*. J. Bacteriol. *141*, 1439–1442.

Ushio, K., Honda, S., Toraya, T., and Fukui, S. (1982). The mechanism of in situ reactivation of glycerol-inactivated coenzyme $B_{12}$-dependent enzymes, glycerol dehydratase and diol dehydratase. J. Nutr. Sci. Vitaminol. (Tokyo) *28*, 225–236.

Wagner, O.W., Lee, H.A., Jr., Frey, P.A., and Abeles, R.H. (1966). Studies on the mechanism of action of cobamide coenzymes. Chemical properties of the enzyme-coenzyme complex. J. Biol. Chem. *241*, 1751–1762.

Weeks, C.M., and Miller, R. (1999). The design and implementation of *SnB* v2.0. J. Appl. Crystallogr. *32*, 120–124.

Yamanishi, M., Yunoki, M., Tobimatsu, T., Sato, H., Matsui, J., Dokiya, A., Iuchi, Y., Oe, K., Suto, K., Shibata, N., et al. (2002). The crystal structure of coenzyme $B_{12}$-dependent glycerol dehydratase in complex with cobalamin and propane-1,2-diol. Eur. J. Biochem. *269*, 4484–4494.

## Accession Numbers

The coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 2D0O (ADP bound form of DDR) and 2D0P (nucleotide-free form of DDR).